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Affidavit

PATENT  
Attorney Docket No.: 023070-095600US  
Client Ref. No.: 1999-158-1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

WILKINS, Thea A.

Application No.: 09/453,387

Filed: December 2, 1999

For: COTTON TRANSCRIPTION  
FACTORS AND THEIR USES

Examiner: Baum, Stuart F.

Art Unit: 1638

DECLARATION OF THEA A. WILKINS  
UNDER 37 C.F.R. §1.132

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

I, Thea A. Wilkins, do hereby state and declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

I am currently Professor in the Department of Agronomy & Range Science at the University of California, in Davis, California. I have worked in the field of plant biology and genetics for 22 years. I have a Ph.D. degree in *Cell and Molecular Biology* from Michigan State University; an M.S. degree in *Plant Genetics* from the University of Georgia; and a *Summa Cum Laude* B.S. degree in *Plant Biology* from Georgia Southern University. A copy of my *Curriculum Vitae* is attached as Exhibit 1.

I have read the Office Action mailed October 3, 2002 and the references cited therein. Although the Examiner acknowledges that the exemplified GhMYB1 gene (SEQ ID NO:1) can be used to modulate transcription, he questions whether other sequences

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within the scope of the claims can also function in this way. As shown by the evidence attached hereto (see Exhibit 2) and discussed below in detail (see Part I, *vide infra*), my lab has isolated the MYB1 gene from *Gossypium arboreum*. As explained below, there are structural similarities between MYB polypeptides from *Gossypium hirsutum* and *Gossypium arboreum*. We have also successfully modulated transcription in a cotton plant using a nucleotide sequence of the invention (see Part II, *vide infra*).

#### I. Identification of Orthologous Gene Sequences for GhMYB1

Based on the GhMYB1 sequence disclosed in the present application, an ortholog was identified from *Gossypium arboreum*, *i.e.*, GaMYB1. We identified an EST (GaMYB1 EST) through a bioinformatics approach by searching an EST database (Ga Cotton Fiber dbEST) with the GhMYB1 gene sequence. The Ga (*Gossypium arboreum*) Cotton Fiber dbEST was a gene discovery project funded by my NSF Cotton Genome Project. Since the EST is only a partial sequence (about 497 nucleotides in length), we isolated the cDNA clone from the bacterial stock and sequenced the clone in its entirety from both ends of the sequence, *i.e.*, from both the 5' and 3' termini. Sequencing the GaMYB1 gene was performed solely by my laboratory and was not part of the NSF Cotton Genome Project. The isolated cDNA clone was found to span 793 nucleotides in length. The GaMYB1 EST cDNA clone is identified by Accession No. Ga\_Eb0006B13 and forms Contig No. COM\_001\_20208.

A translation and sequence alignment of GaMYB1 and GhMYB1 shows a 98% amino acid match in the highly conserved DNA-Binding Domain (DBD) of the two polypeptides (see Exhibit 2). On page 25, lines 1-4 of the specification we indicate that the critical region spanning the DBD is conserved among all six cotton GhMYBs, with amino acid identities ranging from a low of 54.8% (GhMYB5 vs. GhMYB6) to a high of 84.6% (GhMYB1 vs. GhMYB6). In my opinion, the high sequence identity between Ga and Gh, and the fact that MYB1 is encoded by a single gene in the cotton genome, is evidence that establishes that GaMYB1 and GhMYB1 are orthologous genes. The *G. arboreum* clone (GaMYB1) is the second MYB1 gene that was isolated after the *G. hirsutum* clone (GhMYB1). The nucleotide and amino acid sequences for GaMYB1 as well as the sequence alignment with GhMYB1 are attached hereto as Exhibit 2.

## II. Phenotypic Changes in Cotton Plant as Evidence for Modulation of Transcription by MYB Proteins

The following evidence shows that cotton fiber properties are altered and improved in transgenic cotton plants expressing the GhMYB1 gene.

A fiber analysis was performed on fiber that was hand-ginned (*i.e.*, separating the cotton fiber from the seed) from untransformed vector controls as well as (fertile) transgenic lines to identify any general trends that suggest that fiber growth and development had been altered in response to changes in GhMYB1 gene expression (*e.g.*, substantial changes in agronomic properties such as yield and quality). Fiber data were collected from a subset of kanamycin-resistant transgenic cotton plants that were confirmed to contain the transgene 35S::GhMYB1. The transgenic plants were prepared according to standard techniques

The fiber data were collected three times from the same sample and reported as the mean. Table 1 below depicts the summary of fiber data from the first generation hemizygous plants (T0) of 35S::GhMYB1 transgenic cotton plants. The micronaire value is a measurement of cotton fiber quality that is a reflection of both fineness and maturity (low values indicate fine and/or immature fiber; high values indicate coarse and/or mature fibers). Generally, good micronaire ranges from about 3.5 to about 3.6. These values were determined according to standard techniques by measuring the resistance offered by a plug of cotton to airflow that is influenced by a combination of fineness and maturity. Fineness is the outside diameter of the fiber that is measured in mTex (Millitex or mg/km). These values were determined by standard techniques in the art. Maturity is the degree of wall thickening of the fiber. Short fiber count (w) % refers to the percentage of short fiber weight; short fiber count (n) % refers to the percentage of short fiber yield. Immature fiber count refers to the number of immature fibers, *i.e.*, fibers in which the thickening of the fiber wall is appreciably less than normal.

Statistical analysis of the data indicated that there is a trend among some transgenic lines for increased fiber weight per seed (*e.g.*, 2SAD13, 2SAD15; see Table 1 below, Short Fiber Count (w) %). In addition, fiber yield per seed increased in each of the same kanamycin-resistant lines relative to regenerated control plants (see Table 1 below; Short Fiber Count (n) %). This trend is consistent with the functional role for

GhMYB1 as a repressor of cell wall hydroxycinnamic acid biosynthesis. In addition, some 35S::GhMYB1 transgenic plants showed shifts in fiber fineness and micronaire (e.g., 2SAD20, 3SAD2, 5SAD2, 5SAD7 and 5SAD8; see Table 1 below). In fact, my lab has shown that seven out of seven analyzed transgenic 35S::GhMYB1 plants show altered fiber yield and/or fiber quality (see Table 1 below), indicating that GhMYB1 plays a regulatory role in determining agronomic properties. The underlying mechanism for this role is likely due, in part, to GhMYB1-mediated regulation of secondary metabolism during cotton fiber development.

**Table 1: Summary of Relevant Fiber Data from T0 35S::GhMYB1 Transgenic Cotton Plants (T0)**

Transgenic Line	Micronaire	Short Fiber Count (w) %	Short Fiber Count (n) %	Fineness (mTex)	Immature Fiber Count
2SAD13	↓ 2.8	↑ 8.2	↑ 26.9	↓ 166	↑ 10.9
2SAD15	↓ 2.6	↑ 6.0	↑ 21.0	↓ 164	↑ 9.3
2SAD20	↑ 4.6	↓ 3.9	↑ 18.2	↓ 166	↑ 7.5
3SAD2	↑ 4.8	→ 4.1	↑ 17.5	→ 181	↑ 8.0
5SAD2	↑ 5.5	↓ 1.8	↓ 7.8	↑ 210	↓ 5.0
5SAD7	↑ 5.0	→ 4.1	→ 16.2	→ 181	↑ 7.8
5SAD8	↑ 4.3	↓ 2.5	↓ 10.1	↑ 207	↓ 4.6
Control	3.2	4.2	16.2	180	7.2

The observed trends shown in Table 1 are expected to be stronger in the next generation of transgenic plants (T1), particularly homozygous T1 plants. The arrows in the table indicate the general direction of trait relative to control plants and bold data depicts major shifts compared to control plants. As indicated above, the data represents the means of three replicated measures collected from the same sample. Generally, the trends show similar patterns. As seen in the table, if one trait goes up, then related traits go up or down as expected and relative to one another in most cases. For example, in 2SAD13, a decrease in micronaire to 2.8 corresponds to an increase in immature fibers, which is to be expected, since very low micronaire indicates immature fibers. Conversely, if micronaire goes up in transgenic lines relative to control plants, the other properties show the reverse trend. This is further exemplified by 5SAD8 with a

micronaire of 4.3 that corresponds to a decrease in immature fibers and an increase in fineness. The two trends observed here suggest that there is sense overexpression in some transgenic plants, and sense co-suppression in others.

As indicated above, these fiber data were collected from early generation plants that are hemizygous and still segregating. In other experiments, we have shown that trends observed in early generations become well established, and more dramatic once there are genetically stable, homozygous lines.

### III. Conclusion

In conclusion, these findings clarify and further underscore the novel features and function of the cotton MYB proteins as described in the specification. The features that identify a cotton fiber MYB protein, including a cotton fiber MYB gene with 80% homology to SEQ ID NO:1 have been identified. The function of a MYB polypeptide, namely the modulation of transcription in a plant, particularly a cotton plant, has been further established through the evidence provided.

28 Feb 2003  
Date

Thea A. Wilkins  
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**Peer-Reviewed Publications in Last 4 Years:**

- Wilkins et al. (2003) Cotton fiber ESTs offer a unique perspective on cellular dynamics during rapid cell elongation. *Drafted, under revision*
- Mishra, R., H.-Y. Wang, N. Yadav, and T.A. Wilkins. (2003) Development of highly regenerable elite Acala cotton (*Gossypium hirsutum* L.) – A step towards genotype-independent regeneration. *Plant Cell Tiss. Org. Cult.*: In Press.
- Senchina, D. S., I. Alvarez, R. C. Cronn, B. Liu, J. Rong, R. D. Noyes, A. H. Paterson, R. A. Wing, T. A. Wilkins, and J. F. Wendel (2003) Rate variation among nuclear genes and the age of polyploidy in *Gossypium*. *Molec. Biol. Evol.*: In Press.
- Wilkins, T.A., R. Mishra, and N. Trolinder. (2003) Cotton Transformation. *In Molecular Methods of Plant Analysis*, J.L. Jackson, R.B. Inman and H.F. Linskens, eds. Invited chapter. In Press.
- Cedroni M.L., R.C. Cronn, K.L. Adams, T.A. Wilkins, and J.F. Wendel (2002) Evolution and expression of *MYB* genes in diploid and polyploid cotton. *Plant Molec. Biol.*: In Press
- Chen, X., T. Kanokporn, Q. Zeng, T.A. Wilkins, and A.J. Woods (2001). Characterization of the V-type H<sup>+</sup>-ATPase in the resurrection plant *Tortula ruralis*: Post-transcriptional gene control of the porteolipid c subunit in response to salinity. *J Expt Bot.*: 53:225-232.
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- Wilkins, T.A., and J.A. Jernstedt (1999) Chapter 9. Molecular genetics of developing cotton fibers. *In Cotton Fibers* (A.M. Basra, ed.). Hawthorne Press, New York. pp. 231-267.
- Schumacher, K., D. Vafeados, M. McCarthy, H. Sze, T.A. Wilkins and J. Chory (1999) The Arabidopsis *det3* mutant reveals a central role for the vacuolar H<sup>+</sup>-ATPase in plant growth and development. *Genes Develop.* 13:3259-3270.
- Loguercio, L.L., H.C Scott, N.L. Trolinder, and T.A. Wilkins. 1999. Hmg-co-A reductase gene family in cotton (*Gossypium hirsutum* L.): unique structural features and differential expression of hmg2 are potentially associated with synthesis of specific isoprenoids in developing embryos. *Plant Cell Physiol.* 40:750-761.
- Loguercio, L.L., J.-Q. Zhang, and T.A. Wilkins (1999). Differential regulation of six novel *MYB*-domain genes defines two distinct expression patterns in allotetraploid cotton (*Gossypium hirsutum* L.). *Molec. Gen. Genet.* 261:660-671.
- Kim, W., C.-Y. Wan, and T.A. Wilkins (1999) Functional complementation of yeast *vma1Δ* cells by a plant subunit A homolog rescues the mutant phenotype and partially restores vacuolar H<sup>+</sup>-ATPase activity. *Plant J.* 17:501-510.